November 11, 1999

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Process for preparing antibodies against a polypeptide, the nucleic acid encoding which is known

Because of the enormous advances which have been made in the possibilities for sequencing nucleic acids, the problem frequently arises in molecular biology that, while the genetic information for a polypeptide or protein is known, this polypeptide or protein is not available in pure form. While nucleotide sequences are continually being published as a result of the Human Genome Project, the functions possessed by the polypeptides or proteins encoded by these genes are frequently completely unknown.

As a rule, it is very helpful for the practical application and evaluation of these scientific findings if these proteins can be detected using suitable antibodies. Such antibodies can be used either to purify the proteins or, for example, to determine the location of the proteins in tissues and cells.

An object of the present invention is therefore to make available antibodies which are directed against polypeptides or proteins, the nucleotide sequences for which are known but which are not available in enriched, and certainly not in purified, form.

Conventionally, antibodies are prepared by the proteins first of all being purified from the cells or the tissue, or being prepared recombinantly using bacteria, or in insect cells or mammalian cells, and these proteins being used for immunizing animals. These methods are frequently very elaborate and long-winded. The proteins which have been prepared in bacteria are frequently not identical to the naturally occurring

proteins since their secondary structure may differ from that of the native proteins and since bacteria do not possess the same post-translational modification mechanisms as those which are present in eucaryotic organisms.

The present invention therefore relates to a process for producing antibodies which react specifically with a polypeptide, the nucleic acid encoding which is known, in which process

a) the DNA encoding the polypeptide is expressed in a host cell using a vector which possesses at least one sequence encoding a detection signal, and the expressed polypeptide is bound to a solid phase with the aid of the detection signal,

b) independently of step a), the DNA encoding the polypeptide is introduced directly into an animal, resulting in expression of a polypeptide in the animal, which expression causes the formation of antibodies against the polypeptide, and

c) the antibodies which are formed in step b) are reacted with the polypeptide formed in step a) and detected or enriched.

The process according to the invention essentially consists of three steps. On the one hand, the DNA encoding the polypeptide is expressed in a suitable host cell using a vector (step a)). Since the polypeptide which is expressed using the vector is as a rule only present at relatively low concentration in the host cell, the vector employed is provided, according to the invention, with a nucleotide sequence which encodes a detection sequence (tag sequence). This tag sequence is linked to the sequence encoding the polypeptide, resulting in the expressed polypeptide possessing this detection peptide sequence at the C terminus, for example.

In step b), which is carried out independently of step a), the DNA encoding the polypeptide is introduced into a suitable animal and expressed in this animal. The genetic immunization which is employed in accordance with the invention enables antibodies to be formed directly in a host animal.

In this method of preparing antibodies, purified DNA, which contains the genetic information for the protein to be investigated and suitable control elements, is injected directly into the organism (mouse, rabbit, etc.) which is earmarked for the antibody production. The DNA is taken up by the cells of the recipient organism and the protein is expressed in native form (i.e. with correct post-translational modifications). The protein, which is foreign as far as the recipient organism is concerned, induces the immune system to produce antibodies which are directed against the foreign antigen (humoral immune response). This method has already been employed successfully for producing high-affinity monoclonal antibodies which recognise native proteins.

The expression vectors which are employed for the genetic immunization in step b), for the purpose of preparing the desired antibodies, are also to be used in vitro for producing the target protein. Transient transfection (electroporation, lipofection, etc.) is used to introduce the expression vectors into suitable target cells, in particular mammalian cells, which then synthesize the desired protein. These cells (intact or following lysis with suitable buffers) or medium supernatants (in the case of secreted proteins) are to be used for detecting the protein-recognizing antibody by means of FACScan analyses (in the case of proteins which are located in the cell) or ELISA.

When a foreign polypeptide is expressed in a host cell, the expressed polypeptide can usually be secreted to the exterior using a secretion sequence or leader sequence. In these cases, it is important that the expressed and secreted polypeptide possesses a

detection signal which can be used to isolate the polypeptide from the medium. If, however, the polypeptide is not secreted to the exterior but remains on the surface of the cell membrane, an additional detection sequence is then not absolutely necessary. In this case, the site in the polypeptide which is responsible for the anchoring between polypeptide and cell assumes the function of the detection sequence. Since, in this case, the expressed polypeptide remains linked to the cell, the antibodies which are formed can be detected by means of FACScan analyses, by binding to the polypeptide and subsequently reacting with a fluorescence-labeled antibody. As an alternative, it is also possible to carry out a cell ELISA in which the bound antibodies are detected using an enzyme-coupled secondary antibody and a suitable substrate reaction. If the anchoring sequence is a signal sequence which is responsible for anchoring to a membrane by way of a glycosylphosphatidylinositol (GPI) residue, the corresponding expression plasmid can then be used both for DNA immunization and for detecting the resulting antigen-specific antibodies, e.g. following transient transfection. The advantage of a GPI anchor is that it is easily cleaved enzymically from the cell surface in vivo and that it is consequently possible to achieve a good antibody reaction, as is known for secreted proteins (see Example 7 for a good immune response following genetic immunization with an expression plasmid which encodes a GPI-anchored protein).

In the case of secreted proteins (where appropriate, also in the case of proteins which are expressed intracellularly), it is necessary to attach a detection sequence (a tag) to the antigen recombinantly. This tag sequence enables the protein to be fished out of the cell supernatant or cell lysate using substances which interact with the tag sequence and which are bound to a solid matrix (e.g. antibodies which recognise the tag sequence; in the case of the His_6 tag sequence, suitable complexed Ni^{2+} ions).

Peptide sequences which are short and/or not very immunogenic are particularly suitable tag sequences. Mouse proteins which have a stimulatory effect on antibody production (e.g. GM-CSF, IL-4, IL-10, etc.) and which at the same time are able to function as tags can be used as tag sequences which are not particularly immunogenic (i.e. for preparing antibodies in mice). Such tags have the advantage of not developing any immune response because of the tolerance of the immunized animal towards these self-proteins. If it is not possible to prevent the formation of antibodies which recognise the tag sequence of a recombinant protein, these antibodies can be identified using constructs which encode irrelevant proteins which are provided with an identical tag.

The immobilized protein, which has been prepared by transient transfection, is now used to bind the antibodies, which recognise it, from the serum or the hybridoma culture supernatant (when preparing monoclonal antibodies). The bound, specific antibodies are then detected using enzyme-coupled anti-antibodies (detection antibodies) which are quantifiable, as a rule photometrically, by way of a specific substrate reaction. When using peptide tags, the specificity and sensitivity of the detection system can be significantly increased if $F(ab)_2$ fragments of the anti-tag antibody are used as captor antibodies and an Fc region-recognising antibody is used as the detection antibody. This configuration of the ELISA rules out any cross-recognition of the captor antibody.

The transcription unit which encodes the polypeptide can have a polyadenylation sequence, which is required for stabilizing a eucaryotic mRNA, at its 3' end.

In order to ensure that the polypeptide is expressed in the host cell, the vector normally possesses a promoter, with preference being given to using strong promoters. Examples which may be mentioned

are the elongation factor 1α promoter or the cytomegalovirus promoter.

In the process according to the invention, the nucleic acid encoding the polypeptide is introduced directly into an animal in order to produce antibodies against the polypeptide in this animal. In a preferred form, the DNA which is employed for this purpose is present in the form of a vector which is selected such that it can be used for the two steps a) and b) at one and the same time. In a particularly preferred embodiment, the polypeptide-encoding DNA is introduced using a so-called gene gun. In the gene gun method, microscopically small gold particles are coated with the DNA, preferably the vector or plasmid DNA, and shot at the shaved skin of the experimental animal. The gold particles then penetrate into the skin and express the DNA which has been applied to them in the host animal. Preference is given, according to the invention, to using laboratory animals such as mice, rats or rabbits.

In order to achieve a more vigorous antibody formation, so-called genetic adjuvants are, in a preferred embodiment, also applied simultaneously with the polypeptide-encoding DNA. These genetic adjuvants are plasmids which express cytokines (such as GM-CSF, IL-4 and IL-10) and which stimulate the humoral immune response in the laboratory animals.

Particularly when the laboratory animal employed is a mouse or a rat, there is the opportunity of forming hybridoma cells. The immunized mice are sacrificed, spleen cells are isolated and fused with tumor cells, and those clones which secrete the desired monoclonal antibodies are then selected.

In a particularly advantageous embodiment, the polypeptides to be investigated are secreted from the host cells in step a). Since a detection signal is linked to the polypeptides, the sought-after polypeptides can be isolated by forming a bond between the detection signal (tag sequence) and a suitable ligand. The tag sequence is preferably bound to a solid

phase. This solid phase can be the walls of microtiter plates, gel spheres or else magnetic beads. Magnetic beads have the advantage that the solution containing the expressed polypeptide can be readily mixed with the magnetic beads. The magnetic beads possess a ligand (for example antibody fragments) which binds to the tag sequence. The magnetic beads can then be concentrated by applying a magnetic field. By choosing suitable conditions, the sought-after polypeptide can then be eluted once again from the magnetic beads when the antibodies are to be enriched.

The present invention also relates to the antibodies which can be obtained using the process according to the invention.

Figure 1 shows the detection, by means of FACScan analysis, of anti-hp70 antibodies in serum and in the culture supernatant from hybridomas obtained from the lymph nodes of mice immunized with hp70-pcDNA3 DNA. BOSC cells which were either untransfected (gray curves) or transiently transfected with hp70-pcDNA3 DNA (white curves) were used for the FACScan analysis. GV114, mouse immunized with the hp70-pcDNA3 expression vector. The experiment is explained in more detail in example 7.

The present invention is explained in more detail with the aid of the following examples.

Example 1

Preparing murine monoclonal antibodies by means of genetic immunization without purified antigen (protein)

a) Expression construct for the genetic immunization

An expression construct based on the commercially available expression vector pcDNA3 (Invitrogen) was selected. In this vector, the cDNA is expressed under the control of the cytomegalovirus (CMV) promoter. However, it is also possible to use

other, preferably strong, usually ubiquitously active promoters (e.g. the promoter of the elongation factor 1α [EL-1α] gene). The human cDNA region encoding the extracellular domain of thyroid peroxidase (TPO) (2602 bp; 859 amino acids) was cloned into the BamHI/EcoRV cleavage sites in the polylinker sequence and additionally provided, at the 3' end, with a region encoding a His_6 tag and a subsequent stop codon: (TPO sol.-His-pcDNA3). The plasmid DNA was replicated in E. coli and purified using a Qiagen plasmid isolation kit (Qiagen, Hilden).

b) Genetic immunization of mice

In principle, there are two different methods for administering DNA for the genetic immunization. These methods are intramuscular injection or intracutaneous administration using gas pressure-accelerated, microscopically small gold particles coated with plasmid DNA (gene gun). We used the gene gun method for the Example. For this, 200 μg of TPO sol.-His-pcDNA3 DNA were applied per 25 mg of gold particles in accordance with the manufacturer's instructions (*gene gun optimization kit;* Bio-Rad, Munich). For the genetic immunization, the abdominal fur (approx. 4 cm^2) was removed, using perfume-free depilation cream (Veet), from five mice after they had been anaesthetized (intraperitonially) with 110 μl of ketamine/xylazine (100 mg/kg/16 mg/kg); the mice were then bombarded twice with the gene gun (Helios gene gun; Bio-Rad). 1 μg of plasmid DNA was administered per "bombardment". The immunization was repeated after 19 days, and blood was withdrawn 14 days later for determining the quantity of specific antibodies.

Example 2

Expressing the protein encoded by the expression construct

The protein encoded by the expression plasmid has to be prepared in order to detect the specific antibodies which are formed as a result of the genetic immunization. In order to obtain the protein in native form (as in the immunized animal), the expression construct was introduced by transfection into BOSC23 cells [Pear et al., (1993) PNAS, 84, 8392-8396]. BOSC23 cells are a modified adenovirus 5-transformed human embryonic kidney cell line (HEK293) which can be transiently transfected very satisfactorily. The cells were plated out in 6-well cell culture dishes such that they reached 80% confluence on the following day. They were then washed three times with in each case 2 ml of serum-free and antibiotic-free *Dulbecco's modified Eagle's medium* (DMEM) medium and treated with 2 µg of expression plasmid/10 µl of lipofectamine (Life Technologies, Eggenstein) in 1 ml of serum-free and antibiotic-free DMEM medium. The DNA/lipofectamine/medium mixture had previously been pipetted together in a polystyrene vessel and incubated for 10 minutes at room temperature. Following a 6-hour incubation at 37°C and 10% CO_2, 2 ml of DMEM/20% fetal calf serum (FCS) were added. 24 h after transfection (corresponds to the time at which the DNA was added), the medium was replaced with 5 ml of DMEM/5% FCS. After a further 48 h (72 h after transfection), the cell culture supernatant was removed and stored at -70°C.

Example 3

Detecting specific antibodies which are directed against the protein encoded by the expression construct

In order to bind the His_6 tag protein (TPO sol.-His) prepared by transient transfection to nickel chelate microtiter plates (DUNN, Asbach), the wells were in each case incubated, overnight at 4°C, with 200 µl of supernatant from the transient transfection mixture (see above) or of a mock-transfected BOSC23

culture supernatant and then washed 4 times with buffer A (50 mM tris/HCl, pH 7.5, 1 M NaCl) and twice with buffer B (phosphate-buffered saline (PBS), 0.1% BSA, 0.05% Tween 20). Nonspecific binding sites were then blocked by incubating with 300 µl of 3% bovine serum albumin (BSA)/PBS at room temperature for 1 h, after which the washes with buffer A and buffer B were repeated. The pre-immune sera and the immune sera from the immunized mice were diluted 1:100 with buffer B. In each case 100 µl of the diluted mouse sera were added to the wells of the nickel chelate microtiter plates. After incubating at room temperature for 1 hour, the wells were in each case washed four times with buffer C (50 mM tris/HCl, pH 7.5, 0.5 M NaCl, 0.1% BSA, 0.05% Tween 20) and twice with buffer B and then treated with 100 µl of rabbit anti-mouse Ig peroxidase conjugate (DAKO, Hamburg) diluted 1:2000 with buffer B. After a one-hour incubation, the wells were washed four times with buffer C and twice with buffer B and in each case treated with 100 µl of 3,3',5,5'-tetramethylbenzidene substrate solution (Fluka, Buchs, Switzerland). After sufficient development, the color reaction was stopped by adding 50 µl of 0.5 M H_2SO_4 and measured in an ELISA reader at a wavelength of 450 nm.

In order to check the serviceability of the invention which is presented here, the specific antibodies directed against TPO were detected "classically" by means of a commercially available TPO antibody ELISA (Varelisa TPO antibody; Pharmacia-Upjohn, Freiburg). In this test system, anti-TPO antibodies are detected using purified recombinant TPO. The content of anti-TPO antibodies in the pre-immune and immune sera from the immunized mice was determined at a dilution of 1:100 in accordance with the manufacturer's instructions.

Results:

It was possible to detect anti-TPO antibodies unambiguously, as compared with the pre-immune sera, at a dilution of 1:100, in the serum obtained from all the five mice which were immunized with TPO sol.-His-pcDNA3 DNA. The results are presented in Table 1.

Table 1: Detection of anti-TPO antibodies in the serum of TPO sol.-His-pcDNA3 DNA-immunized mice using purified TPO protein (*Varelisa TPO Antibodies* detection system).

Mouse	Optical density$_{450\ nm}$	
	Pre-immune serum	Immune serum
GV1	0.09	2.53
GV2	0.06	1.97
GV3	0.07	1.13
GV4	0.08	1.63
GV5	0.08	0.60

The detection system according to the invention was used to investigate the pre-immune serum and immune serum from a mouse (GV1 in Table 1) as an example. As can be seen from Table 2, it is possible to detect anti-TPO antibodies unambiguously, at a serum dilution of 1:100, in the immune serum whereas the pre-immune serum did not exhibit any reaction.

Table 2: Detection of anti-TPO antibodies in the serum of a TPO sol.-His-pcDNA3 DNA-immunized mouse using TPO sol.-His protein which was produced by transient expression.

Serum or buffer	Dilution with buffer A	Optical density TPO sol.-His	Medium
pre-immune	1:100	0.17	0.15
immune	1:100	0.55	0.19

buffer A	--	0.03	0.01

Example 4

Preparing polyclonal antibodies by means of genetic immunization without purified antigen (protein) in rabbits

a) Expression construct for the genetic immunization

For the second case example, the ubiquitously active puomoter of the elongation factor 1α (EF-1α) gene was used for controlling the expression. The expression vector employed is based on the pBluescript vector (Stratagene, Heidelberg), into which a 1.2 kb fragment of the human EF-1α gene promoter, an 0.7 kb EcoRI fragment containing the polyadenylation signal from the cDNA for human G-CFS (Mizushima and Nagata, 1990), and also, between the BamHI and NotI cleavage sites, the oligonucleotide sequence encoding the influenza virus hemagglutinin (HA) tag were incorporated. The human cDNA region encoding the extracellular domain of the activin receptor IIA (431 bp; 135 amino acids) was cloned into the ClaI/BamHI cleavage sites of the polylinker sequence such that the HA tag-encoding region and a subsequent stop codon came to lie at the 3' end (pEF-1α-ActRII-HA).

b) Genetic immunization of rabbits

For the genetic immunization, 100 µg of pEF-1α-ActRII-HA DNA were applied per 25 mg of gold particles in accordance with the manufacturer's instructions (*gene gun optimization kit;* Bio-Rad, Munich). After having been anaesthetized with 15 mg of pentobarbital/kg and having 200 cm^2 of the abdominal fur depilated with depilation cream, two rabbits (Chinchilla Bastard; Charles River, Sulzfeld) were

bombarded 30 times with the gene gun. 1 μg of plasmid DNA mixture was administered per "bombardment". The immunization was repeated after 21 days and blood was removed 21 days later for determining the quantity of specific antibodies.

Example 5

Expressing the protein encoded by the expression construct

The protein encoded by the expression plasmid pEF-1α-ActRII-HA was prepared, as described in Example 2, by transiently transfecting BOSC23 cells.

Example 6

Detecting specific antibodies which are directed against the protein encoded by the expression construct

In order to bind the HA tag protein (EF-1α-ActRII-HA), prepared by transient transfection, to microtiter plates, the wells were first of all coated with the $F(ab)_2$ fragment of the anti-HA tag antibody. For this, 150 μl of the antibody fragment were added to each well of the microtiter plate, after which the plate was washed with PBS at room temperature and free protein-binding sites were blocked by incubating with 200 μl of 0.2% BSA/PBS/well.

The supernatant from the transient transfection mixture (see Example 5), or from a mock transfected BOSC23 culture supernatant[sic], was then incubated at room temperature for 2 h, after which the plates were washed three times with phosphase-buffered saline (PBS). The pre-immune sera and immune sera from the immunized rabbits were diluted 1:100 and 1:500, respectively, with 0.2% BSA/PBS. 100 μl of the diluted rabbit sera were in each case added to the wells of the coated microtiter plates. After the plates had been

incubated at room temperature for one hour, the wells were in each case washed three times with PBS, after which 100 µl of goat anti-rabbit Ig peroxidase conjugate (DAKO, Hamburg), diluted 1:2000 with PBS/0.2% BSA) were added to each well. After the plate had been incubated for one hour, the wells were washed three times with PBS, after which 100 µl of 3,3',5,5'-tetramethylbenzidene substrate solution (Fluka, Buchs, Switzerland) were added to each well. After it had developed sufficiently, the color reaction was stopped by adding 50 µl of 0.5 M H_2SO_4 to each well and measured in an ELISA reader. The results showed that it is also possible to use the process according to the invention to produce specific polyclonal antibodies against an unknown gene product in rabbits.

Example 7

Using genetic immunization to prepare murine monoclonal antibodies against a human GPI-anchored surface protein

a) Expression construct for the genetic immunization

For the genetic immunization, the complete hp70 cDNA, encoding a 70 kDa GPI-anchored human surface protein, was cloned into pcDNA3 (hp70-pcDNA3) and replicated (see Example 1). Approx. 70% of the residues of the human hp70 amino acid sequence tally with those of the murine hp70 sequence.

b) Genetic immunization of mice

The mice were immunized with the gene gun (see Example 1b) using a short protocol (6 immunizations within 13 days), as described by Kilpatrick et al. (1998), Hybridoma 17: 569-576.

c) Preparing hybridomas for producing monoclonal antibodies

In order to prepare hybridomas, lymphocytes were isolated from the regional (axillary, brachial, inguinal and popliteal) lymph nodes from three mice and fused, in accordance with a standard protocol, with exponentially growing SP2/0 mouse myeloma cells (American Tissue Type Culture Collection) using polyethylene glycol (Sigma) (Campbell A M (1986). Monoclonal antibody technology: The production and characterization of rodent and human monoclonal antibodies. Book series: Laboratory Techniques in Biochemistry and Molecular Biology (R H Burdon and P H van Knippenberg, eds.), Elsevier Science Publishers, Amsterdam). 2 x 10^5 fused lymph node lymphocytes were plated out in each well of a 96-well microtiter plate and in each case cultured in 100 µl of hypoxanthine/aminopterin/thymidine (HAT)-containing DMEM medium (Sigma) containing 20% FCS and 5% *Hybridoma Enhancing Factor* (Sigma).

d) Detecting specific antibodies using cells in which the expression construct used for the genetic immunization is expressed following transient transfection

Candidate hybridoma clones were identified by means of a cell ELISA. For this, BOSC cells, as described in Example 2, were transiently transfected with the hp70-pcDNA3 expression construct, resuspended in 4% formaldehyde in PBS and fixed for 10 min. The cells were then diluted 1:10 with PBS and stored at 4°C for up to four weeks.

Cell ELISA

96-well round-bottom microtiter plates were blocked at room temperature by adding 300 µl of 1% BSA in PBS per well for 1 h. After the solution had been removed by inverting the plates, 75 µl of the hybridoma

cell supernatant and 10 µl of transiently transfected BOSC cell suspension (6 x 10^6 cells/ml of 1% BSA in PBS) were added per well and the plates were incubated at 4°C for 1 h. After 100 µl of 1% BSA in PBS had been added to the well, the plates were centrifuged at 300 x g for 4 minutes and the supernatants were tipped out as above. The cells were washed once again with 200 µl of 1% BSA/PBS/well, resuspended in each case in 75 µl of peroxidase-coupled goat anti-mouse immunoglobulin antibody (DAKO), diluted 1:2000 in 1% BSA/PBS, and incubated at 4°C for 1 h. 100 µl of 0.1% Tween 20/PBS were then added per well and the plates were centrifuged as above and the supernatants discarded. The cells were then washed three times with in each case 200 µl of 0.1% Tween 20/PBS and twice with in each case 200 µl of PBS. Peroxidase-coupled goat anti-mouse IgG antibody (diluted 1:2000) or goat anti-mouse IgM antibody (diluted 1:2000) (Southern Biotechnologies Associates) was used to determine the immunoglobulin class (IgG or IgM) of the monoclonal antibodies in the hybridoma supernatants. The peroxidase bound to the cells by way of the antibodies was quantified by adding 3,3',5,5'-tetramethylbenzidine substrate solution as described in Example 3.

Results:

In all, 176 hybridoma-covered microtiter wells were obtained using the above-described fusion. 64 supernatants from these wells proved to be positive for anti-hp70 antibodies when an OD_{450} value which was twice as high as the blank value obtained with medium (blank value: 0.035) was used as the threshold value. Table 3 lists the values which were measured for a negative (N1B10) hybridoma supernatant and for a positive (N1F4) hybridoma supernatant. The OD values obtained in the same test for the immune serum and pre-immune serum from a mouse (GV114) used for preparing the hp70 hybridoma are shown for comparison. The same N1B10 and

N1F4 hybridoma supernatants were also tested for the presence of specific anti-hp70 antibodies by means of a FACScan (*fluorescence-activated cell scanning*) analysis (see below).

Table 3: Use of a cell ELISA to detect anti-hp70 antibodies in serum and in culture supernatants from hybridomas obtained from the lymph nodes of mice immunized with hp70-pcDNA3 DNA. BOSC cells which were transiently transfected with hp70-pdDNA3 DNA were used for the cell ELISA.

Serum or hybridoma supernatant	Dilution	Optical density$_{450\ nm}$
Pre-immune serum GV114	1:100	0.08
Immune serum GV114	1:100	1.21
Hybridoma supernatant N1B10	undiluted	0.05
Hybridoma supernatant N1F4	undiluted	1.07

FACScan analysis

In each case 10 µl of the suspension of fixed, transiently transfected BOSC cells (20 x 10^6 in 3% FCS/PBS), as described under cell ELISA, were pipetted into the wells of a 96-well round-bottomed microtiter plate, after which 75 µl of the given hybridoma supernatant were added. As controls, cells were treated either with 25 µl of pre-immune or immune sera diluted 1:100 with 3% FCS/PBS or with 25 µl of a control monoclonal antibody (50 µg/ml of 3% FCS/PBS). After the plate had been incubated at 4°C for 30 min, 200 µl of 3% FCS/PBS were added to each well and the cells were centrifuged down, as described above, and the supernatants discarded. After the plate had been washed once with 200 µl of 3% FCS/PBS per well, 25 µl of a phycoerythrin-coupled goat anti-mouse immunoglobulin antibody (Southern Biotechnologies Associates), diluted 1:50 with 3% FCS/PBS (final concentration: 10 µg/ml),

were added per well and the plate was incubated at 4°C for 30 min. The cells were subsequently washed twice as above and the fluorescence was measured in a FACScan appliance (Becton Dickinson).

Results:

20 supernatants giving OD_{450} values of >0.2 were selected from the hybridoma supernatants judged to be positive in the cell ELISA (see above) for the determination of anti-hp70 antibodies by means of FACScan analysis. Figure 1B shows the histograms which were obtained, using BOSC cells which were transiently transfected with the hp70-pcDNA3 expression vector or which were not transfected, for an irrelevant antibody (26/3/13), used as negative control, and for the positive hybridoma supernatant N1F4. The histograms which were obtained in the same test for the immune and pre-immune sera from a mouse used for the hybridoma preparation are shown for comparison (Figure 1A). All the 20 hybridoma supernatants selected proved to be positive in the FACScan analysis. The immunoglobulin class of the hp70-specific antibodies was determined in 19 out of the total of 20 supernatants. Two of the tested supernatants contained hp70-specific IgM antibodies and 17 supernatants contained hp70-specific IgG antibodies.